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The Large Diverse Gene Family *var* Encodes Proteins Involved in Cytoadherence and Antigenic Variation of Plasmodium falciparum-Infected Erythrocytes

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Summary

The human malaria parasite Plasmodium falciparum evades host immunity by varying the antigenic and adhesive character of infected erythrocytes. We describe a large and extremely diverse family of P. falciparum genes (*var*) that encode 200–350 kDa proteins having the expected properties of antigenically variant adhesion molecules. Predicted amino acid sequences of *var* genes show a variable extracellular segment with domains having receptor-binding features, a transmembrane sequence, and a terminal segment that is a probable submembrane anchor. There are 50–150 *var* genes on multiple parasite chromosomes, and some are in clustered arrangements. *var* probes detect two classes of transcripts in steady-state RNA: 7–9 kb *var* transcripts, and an unusual family of 1.8–2.4 kb transcripts that may be involved in expression or rearrangements of *var* genes.

Introduction

Antigenic variation, defined as changes in exposed antigenic determinants at frequencies much higher than the underlying mutation rate, is one of the major means by which bacteria and protozoa can maintain persistent infection in the presence of continual immune attack (Bloom, 1979). Well-known examples are the variations in the pilins and P.II proteins of Neisseria gonorrhoeae, the variations in the major proteins of Borrelia hermsii, and the switches of variant-specific surface glycoproteins of the African trypanosomes (reviewed by Borst and Greaves, 1987; Seifert and So, 1988). In these organisms, antigenic switching occurs principally by DNA rearrangements that move gene sequences from larg repertoires of silent copies into active expression sites. The xpression sit s may themselves also change and may be associated with recombination events that generate novel antigenic forms. As a result, the organisms are capable of producing vast serotype diversity that enables them to deflect immune recognition and maintain infection.

Malaria is a persistent diseas caused by Plasmodium protozoa that propagate within and destroy host erythrocytes. Infections last for periods of months to years, reexposure leads to recurrent infections, and immunity, especially in children, is slow to develop (Hommel, 1985; Miller et al., 1994). Simian malarias were the first to provide clear evidence for antigenic variation at the surface of infected erythrocytes (Brown and Brown, 1965; Brown, 1973; Barnwell et al., 1983; Handunnetti et al., 1987). For Plasmodium falciparum, the cause of the most serious human malaria, direct evidence of antigenic variation was reported from experimentally infected Saimiri monkeys (Hommel et al., 1983). This was complemented by a study that found an extreme degree of antigenic diversity in the parasitized erythrocytes of Gambian children naturally infected with P. falciparum (Marsh and Howard, 1986). In vitro, cloned P. falciparum lines have been found spontaneously to switch their expression of antigenic and cytoadherence types at the surface of infected erythrocytes (Roberts et al., 1992; Biggs et al., 1992).

P. falciparum–infected erythrocytes avoid splenic clearance by adhering to postcapillary venular endothelium, thereby producing sequestration and interference with circulation that may lead to organ-specific damage and the lethal syndrome of cerebral malaria (MacPherson et al., 1985; Pongponratn et al., 1991). The points of attachment to endothelium, electron-dense protrusions of the erythrocyte membrane termed knobs (Luse and Miller, 1971), bind antisera in a variant-specific manner (Langreth and Reese, 1979). Strain-specific antibodies have been found to block binding of homologous but not heterologous parasites to melanoma cells in vitro (Udeinya et al., 1983), suggesting that antigenically variant molecules on the cell surface are responsible for adherence. Indeed, several studies have found that P. falciparum parasites express members of a 200,000–350,000 M_r protein family (PfEMP1) at the infected erythrocyte surface and that these proteins mediate both antigenic variation and cytoadherence (Leech et al., 1984; Magowan et al., 1988; Howard et al., 1988; Biggs et al., 1992). The variability of these proteins in cytoadherence is underscored by the findings that parasitized erythrocytes bind to such diverse receptors as CD36 (Barnwell et al., 1985; Ockenhouse et al., 1989; Oquendo et al., 1989), thrombospondin (Roberts et al., 1985), intercellular adhesion molecule 1 (ICAM-1) (Berendt et al., 1989), endothelial leukocyte adhesion molecule 1 (ELAM-1), and vascular cell adhesion molecule 1 (VCAM-1) (Ockenhouse et al., 1992) and that individual parasites exhibit different profiles of binding to these receptors (Ockenhouse et al., 1991; Biggs et al., 1991; Roberts et al., 1992; Chaiyaroj et al., 1994).

Expression of many alternative 200,000–350,000 M_r proteins of diverse adherence and antigenic properties suggests that these proteins are encoded by a large family of variant genes in P. falciparum. Analogy with antigenic variation in other organisms further suggests that DNA rearrangements within thes genes and alterations in

gene expression sites are mechanisms by which the parasites generate different serotypic forms. Here we describe a large family of P. falciparum genes (*var*) that was identified during analysis of a 300 kb segment of chromosome 7 linked to chloroquine resistance in a P. falciparum cross (Wellems et al., 1991). Characterization of this family and consolidation of findings in this work with those of accompanying reports (Smith et al., 1995; Baruch et al., 1995 [both in this issue of *Cell*]) indicate that *var* genes encode proteins of the PfEMP1 family and modulate the antigenic variation and cytoadherence of P. falciparum–infected erythrocytes.

Results

Identification of a Large Hypervariable Region within a Chromosome 7 Segment Linked to Chloroquine Resistance

Four overlapping yeast artificial chromosomes (YACs) from the P. falciparum FCR3 line were obtained that span the 300 kb chromosome segment linked to CQR, a segment located 300–600 kb from the telomere of chromosome 7. Figure 1 shows the positions of these YACs (PfYEF2, PfYFE6, PfYKF8, and PfYED9) relative to the chromosome map. To define the structure of this 300 kb segment, we performed comparative hybridizations to search for polymorphisms among parasite lines. Clones were randomly picked from chromosome segment–specific plasmid libraries, and their inserts were hybridized against restriction digests of the YAC and parasite DNAs. Over 30 inserts were identified that recognized PfYEF2, PfYFE6, or PfYKF8 and showed a predominance of single-copy sequences with few polymorphisms (AluI, HinfI, RsaI, and SspI digests), consistent with prior findings that chromosome internal regions are largely conserved and contain a preponderance of single-copy sequences (Sinnis and Wellems, 1988; Corcoran et al., 1988; Lanzer et al., 1993). However, 15 other inserts that recognized PfYED9 showed highly polymorphic s ts of repetitive elements in the parasite DNAs (see Figure 2; data not shown). Southern blot analysis indicated that these polymorphic elements were part of a chromosome hypervariable region contained within the PfYED9 clone.

Mapping and DNA Sequencing of the Hypervariable Region Spanned by YAC PfYED9

Single-copy sequences detected by pE45b and pH270.5 flank the hypervariable region spanned by PfYED9 (Figure 1). The pE45b and pH270.5 probes were therefore used to assign large restriction fragments on the PfYED9 map and establish enzyme recognition sites as reference points. A detailed restriction map of the PfYED9 hypervariable region was then developed. Using a chromosome walking approach, we isolated 15 overlapping clones (a–f and h–o in Figure 1) from Dd2 chromosome subsegment libraries (Wellems et al., 1991). The inserts yielded 19.1 kb of continuous Dd2 sequence having predicted enzyme recognition sites in perfect accord with the PfYED9 restriction map. Such agreement indicates that the Dd2 and FCR3 sequences in this part of the chromosome are very similar, despite differences elsewhere in the genome that are evident from restriction analysis (Figure 2).

We also obtained genomic sequence data from the 34 kb ApaI–SmaI fragment of PfYED9. Purified PfYED9 DNA was cut with SmaI to yield a 110 kb fragment, which was then isolated by pulsed-field gel (PFG) electrophoresis and digested with ApaI. The resulting 34 kb ApaI–SmaI band was purified by PFG electrophoresis, digested in four separate reactions by AluI, HinfI, RsaI, or SspI, and incorporated into a plasmid (pCDNAII) library. Cloned inserts from the library were checked for hybridization to the PFYED9 34 kb fragment, assigned to the PfYED9 map, and sequenced (see Figure 1). Overlapping inserts were obtained by the chromosome walking approach except for three gaps (t, z, and θ in Figure 1) that were closed by polymerase chain reaction (PCR) amplification of PfYED9

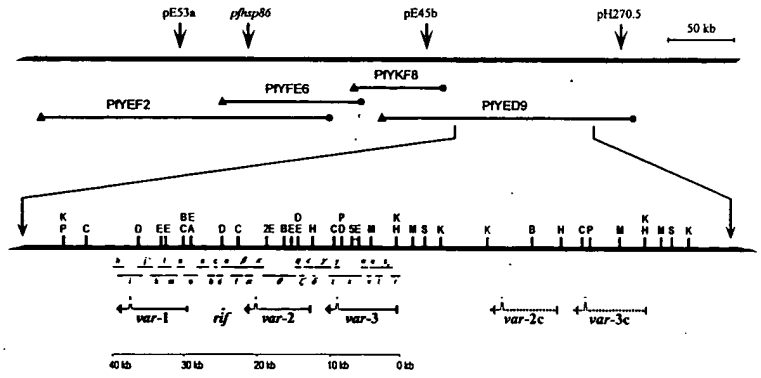


Figure 1. Map of a *var* Gene Cluster on P. falciparum Chromosome 7

Relative positions of four YACs (PfYEF2, PfYFE6, PfYKF8, PfYED9) are indicated under the chromosome 7 line at the top of the figure. YACs PfYFE6 and PfYKF8 lie entirely within a segment linked to CQR in a genetic cross, whereas YACs PfYED9 and PfYEF2 extend beyond sites (identified by pE53a and pH270.5) that are dissociated from the chloroquine response (Wellems et al., 1991; unpublished data). The *var* cluster extends over a region of 100–150 kb in PfYED9. Exons and introns of the *var-1*, *var-2*, and *var-3* genes within the sequenced 40 kb segment are represented by solid and dotted lines, respectively; arrows show the coding direction. Two more *var* elements outside of the sequenced region, identified by conserved restriction sites and cross-hybridization, are indicated by broken lines (*var-2c* and *var-3c*). Bold letters mark repeated restriction sites that suggest a duplication in the *var-2–var-3* and *var-2c–var-3c* segments. Enzyme recognition sites are as follows: A, ApaI; B, BglI; C, ClaI; D, HindIII; E, HaeIII; H, BssHII; K, KpnI; M, BamHI; P, HpaI; S, SmaI. HindIII and HaeIII sites outside of the sequenced region were not mapped. Positions and sizes of inserts from the Dd2 subsegment library are indicated: a, pE280b; b, pB20.3; c, pB600; d, pE21b; e, pB20.24; f, pE32b; h, pE241a; i, pE240a/51d; j, pE33a; k, pB20.23; l, λ17BA6; m, pB20.26; n, pB20SU.27; o, p15J2J3. Inserts from the PfYED9 34 kb ApaI–SmaI fragment library are as follows: r, pB3; s, p3G11; t, pJVs; u, p2E10; v, p1G3; w, p2E3; x, p2B6; y, pE10; z, pJYr; α, pC5; β, p1A3; γ, p1F6; δ, p3C3; ε, pA2; ζ, p2A9; η, p3C4; θ, pJZn; κ, p3D8.

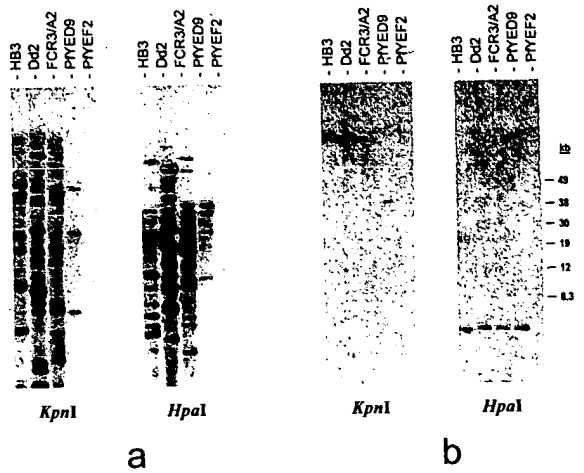


Figure 2. *var* Polymorphisms Detected in Three Lines of P. falciparum and the PfYED9 YAC

DNAs were digested with KpnI or HpaI and separated by PFG electrophoresis.

(a) Signals from restriction fragments probed with the λT147 insert that recognizes *var* exon II. Numerous fragments are evident in the unique patterns from the different parasite lines. Note that the probe detects restriction fragments from PfYED9 but not from PfYEF2.

(b) Single hybridization signals from the pE45b probe confirm complete restriction of the DNAs and balanced loading. A smaller fragment is evident in the PfYED9 lane, because the YAC DNA does not span the full KpnI fragment. Comparable amounts of DNA in the PfYEF2 lanes were confirmed by separate probing with the *pfhsp86* probe (data not shown). Parasite lines were maintained and verified by fingerprint analysis as described by Dolan et al. (1993).

DNA using primers from flanking sequences. The clones from PfYED9 (r–z, γ–κ, and α plus β in Figure 1) yielded 22.2 kb of continuous DNA sequence that overlaps the Dd2 sequence at the f/β junction and has predicted restriction sites that match the PfYED9 map perfectly. The composite sequence from the Dd2 and PfYED9 segments is 40,171 kb.

Structure of a *var* Gene Cluster and Comparative Analysis of Predicted Amino Acid Sequences

The 40,171 bp sequence contains three 10–12 kb regions that have related sequences and structure. Each of these regions harbors a pair of open reading frames (ORFs). The first ORF in each pair begins with a consensus ATG start codon preceded by typical P. falciparum noncoding sequence of abundant AT content (Saul and Battistutta, 1988). The ORFs of each pair are separated by an intervening AT-rich and noncoding sequence of 0.9–1.1 kb. Presence of consensus intron–exon splice junction sequences at either end of these intervening sequences and lack of a consistent translation start site in the 3′ ORF indicate that the each pair of ORFs belongs to an individual gene having a two-exon structure. This has been verified by comparison of the genomic sequences with the cDNA sequence of an expressed gene (*var-7*; subsequent section). The three 10–12 kb regions thus contain members of a variant gene family that have coding regions of 9.23 kb (*var-1*), 7.99 kb (*var-2*), and 9.01 kb (*var-3*) as depicted in Figure 1. Predicted molecular masses of the encoded proteins are 350 kDa, 302 kDa, and 344 kDa, respectively.

Th *var-1*, *var-2*, and *var-3* genes are flanked by additional members of the *var* family in PfYED9. Restriction analysis identified two additional genes that are 12–35 kb upstream of the sequenced region and are closely related to *var-2* and *var-3* (*var-2c* and *var-3c*; see Figure 1). The *var* genes thus have a clustered arrangement in which many individual members are organized in head-to-tail fashion. Between *var-1* and *var-2* is a 5 kb DNA sequence that harbors a short ORF homologous to that of a repetitive element (*rif*) suggested by Weber (1988) to be a transposable element in P. falciparum.

The deduced protein sequences of the *var* genes are highly diverse, yet all contain certain conserved motifs and common structural features (Figures 3 and 4). Database searches identified two to four domains within each *var* sequence that are homologous to cysteine-rich domains of certain Plasmodium molecules involved in the erythrocyte invasion, the P. falciparum EBA-175 erythrocyte-binding protein and the P. vivax and P. knowlesi Duffy antigen–binding proteins (DABP; Adams et al., 1992). In EBA-175 and the DABPs, these cysteine-rich domains contain functional binding regions that determine invasion specificity (Chitnis and Miller, 1994; Sim et al., 1994). We have therefore termed the homologous domains Duffy binding–like (DBL) after the cysteine-rich domain of the P. vivax receptor. In the *var* ORFs, the first domain near the N-terminus (DBL domain 1) is the most conserved of the DBL domains and has amino acid signatures that differentiate it from subsequent domains (e.g., consensus peptide sequences GAcAp[Y/F]rrL, CTxLARsfadlgdiVrgrdLYLG, and VPTYFDYVpqylrwF; Figure 4). Between DBL domains 1 and 2 is another type of conserved domain, a cysteine-rich interdomain region (CIDR) of 300–400 amino acids. The CIDR does not have all the motifs of a DBL domain, but it does have a region at the 3′ end that is homologous to the end of the F1 DBL domain in P. falciparum EBA-175 (asterisks in Figure 4). The conservation evident in the sequences of DBL domain 1 and the CIDR suggest that these regions maintain important structures in the head of the variant molecule.

DBL domains 2, 3, and 4 (numbering is according to *var-1*, the first sequence completed) have less discriminating signatures than domain 1 and show features of cross-alignment and variation in number that suggest these do-

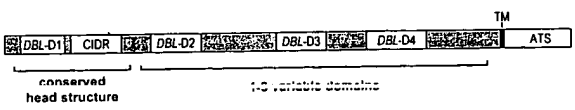
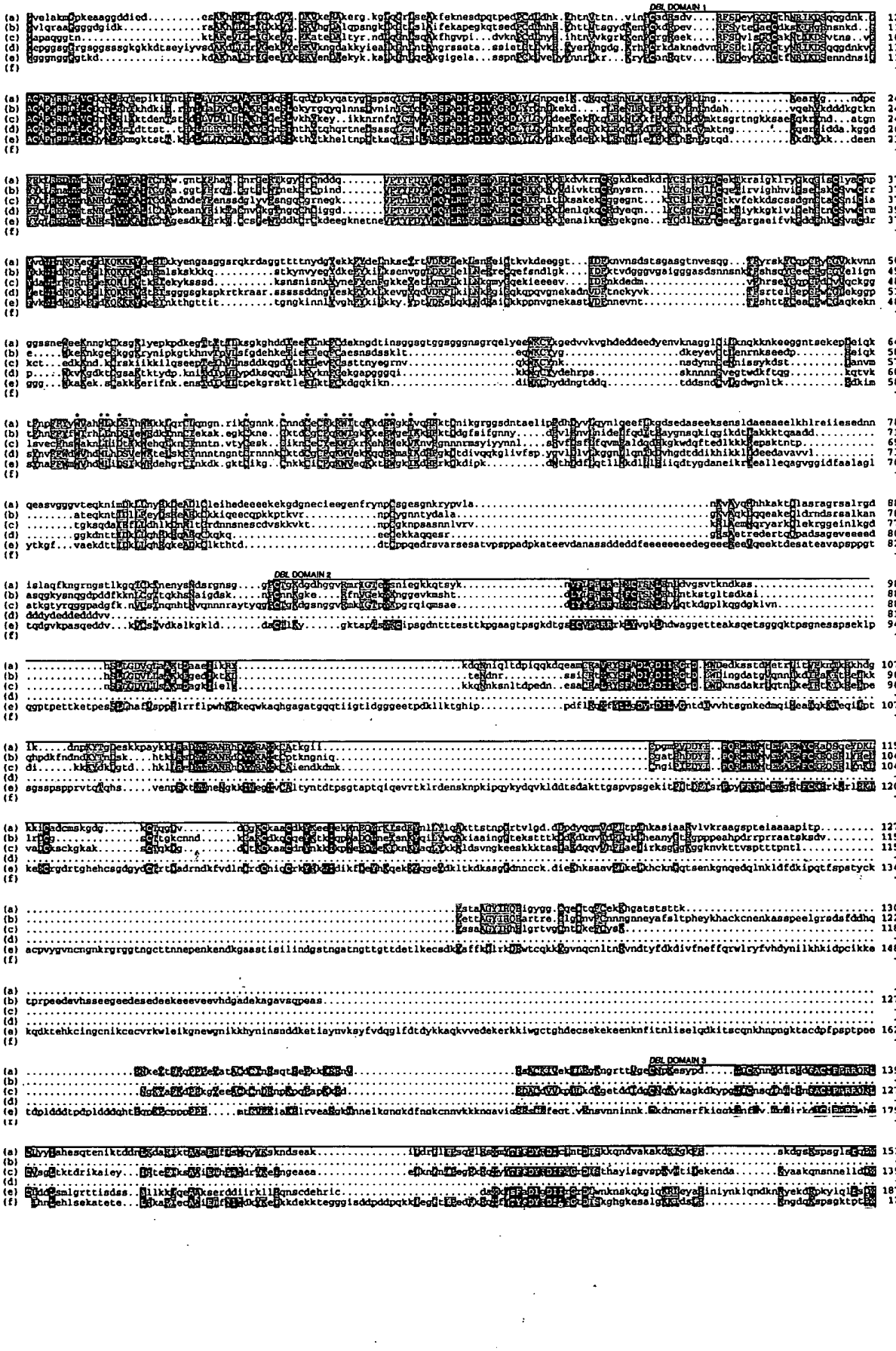


Figure 3. Schematic Diagram of the Variant Molecule Encoded by *var* Genes

Two to four DBL domains and a relatively conserved CIDR are present in the extracellular region of the molecule. The first DBL domain (DBL-D1) and the CIDR may together form a conserved head structure. The transmembrane segment (TM) and a highly charged acidic terminal segment (ATS) may anchor the variant molecule at the knob structure of the infected erythrocyte.


DBL DOMAIN 1
DBL DOMAIN 2
DBL DOMAIN 3

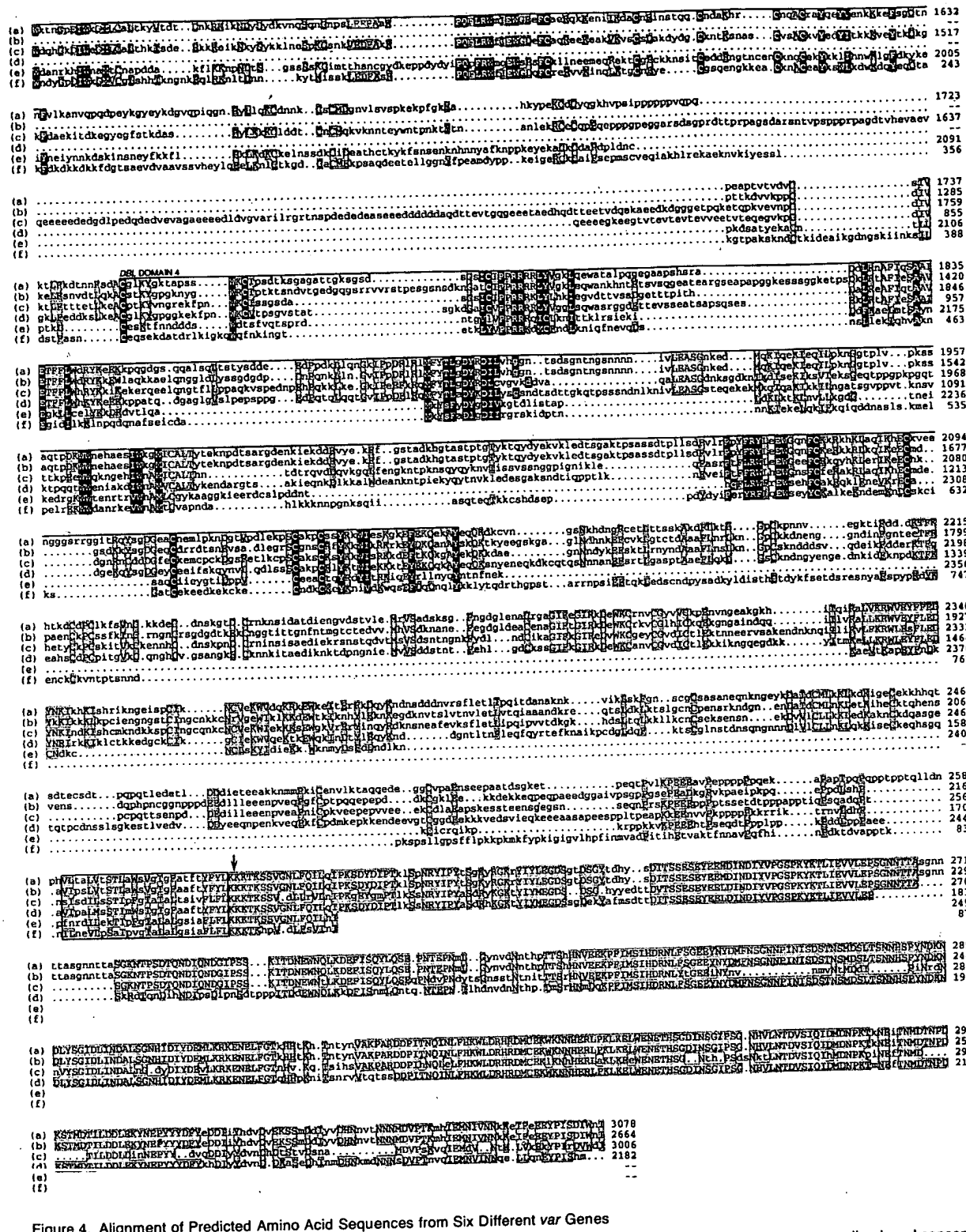


Figure 4. Alignment of Predicted Amino Acid Sequences from Six Different *var* Genes

Identification of DBL domains 1–4 is based upon the *var-1* sequence. DBL domains 1–4 of the predicted sequences are overlined, and conserved amino acids found within all DBL domains are highlighted in reverse. Conserved residues of the CIDR that also occur in the 3′ end of the DBL-F1 domain of P. falciparum EBA-175 are marked by asterisks. Transmembrane sequences (boxed) occur just prior to the junction (downward arrow) between the segments encoded by exons I and II. Preceding the transmembrane segments are proline-rich sequences of variable length. Sequences are from the following: a, *var-1*; b, *var-2*; c, *var-3*; d, *var-7*; e, *MCvar-1* (partial); f, *A4var* (partial). Consensus amino acids identified by GCG sequence analysis software are indicated in shaded capital letters.

mains can undergo shuffling and deletion (Figure 4; analysis not shown). For example, domain 2 of a *var* ORF from the P. falciparum Malayan Camp knob protrusion–positive (MC[K^+]) line (*MCvar-1*, Figure 4) resembles *var-1* domain 4 more than it does *var-1* domain 2, and *var-2* and *var-7* are missing regions corresponding to domain 3 and domains 2 and 3, respectively.

DBL domain 4 is followed by a segment of variable length and a hydrophobic region that is encoded at the end of the first exon (exon I). In all *var* sequences, this hydrophobic region fits the criteria of a transmembrane segment (Engelman et al., 1986). The second exon (exon II) encodes a large (45–55 kDa) conserved C-terminal sequence that has an acid character (predicted pI $\approx$ 4.5, versus 5.9 for the part of the protein upstream of the splice junction) and a cysteine content of <1% (versus >4% upstream). The position of this C-terminal sequence downstream of a single transmembrane segment suggests that it has a cytoplasmic location.

No consensus signal sequence was detected in the N-terminal region of the predicted *var* ORFs. We note the presence of several motifs in the protein sequences that are known to act as ligands and receptors in the integrin family (Haynes, 1992). These include RGD (*var-1* codons 886–888, 1992–1994; *var-2* codons 1577–1579; *MCvar-1* codons 1212–1214) and DGEA (*var-1* codons 2111–2114). Not all of these motifs occur in each protein sequence, and when they do occur, their positions vary.

Identification of *var* Transcripts and Chromosome Expression Sites

To identify transcribed *var* sequences, we screened a λgt10 Dd2 cDNA library with *var*-containing BssHII restriction fragments that had been purified from PfYED9 and radiolabeled by random hexamer priming. This screening yielded 18 clones with inserts that hybridized back to PfYED9. According to cross-hybridization studies and DNA sequence analysis, the inserts fell into two groups: group I inserts that aligned with sequences of *var* exon I (λT240, λT242, λT244, λT284, λT287, λT288, λT295, λT296) and group II inserts that aligned with sequences of *var* exon II (λT140, λT141, λT142, λT145, λT147, λT148, λT150, λT152).

The full ORF of an expressed *var* gene (*var-7*) was determined from λT242 and overlapping cDNA clones that were obtained by a PCR-based walking strategy (Su and Wellems, 1994). The sequence showed that *var-7* has a 6.6 kb ORF containing two DBL domains, a hydrophobic transmembrane sequence, and C-terminal region typical of *var* genes (predicted molecular mass, 249 kDa). Comparison of *var-7* with the *var-1*, *var-2*, and *var-3* sequences demonstrated continuity of the alignments at the predicted splice junction between the ORFs of exons I and II (Figure 4). PCR amplification of Dd2 genomic DNA was also performed with primers derived from the two *var-7* exons. Sequence of this *var-7* PCR product confirmed consensus splice sites and a 1 kb intron typical of the *var* genes. Transcription of *var-7* was detected as a 7.5 kb band by RNA blot analysis (Figure 5a).

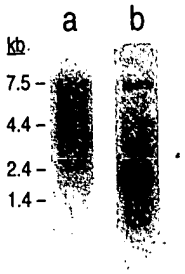


Figure 5. Detection of *var-7* mRNA and a Family of 1.8–2.4 kb Transcripts Related to *var* Exon II

(a) Signal from Dd2 RNA (mixed erythrocytic stage parasites) probed with the λT242 cDNA insert (*var-7* sequence 903–2975 nt from exon I). A 7.5 kb transcript is detected.

(b) Signals obtained after probing with a cDNA insert (λT152) that hybridizes to exon II of the *var-7* gene. The 7.5 kb transcript is detected as well as another diffuse band of 1.8–2.4 kb transcripts. RNA size standards are indicated in kilobases.

Chromosome mapping experiments with a *var-7*-specific probe localized the *var-7* gene to a region that is 600 kb from one end of Dd2 chromosome 12 (chromosome 12 has a length of 2600 kb; data not shown). No hybridization of the *var-7* probe was detected to any other Dd2 chromosome nor to any chromosomes of the HB3, 3D7, or A4 parasites. Other cDNA inserts from the group I clones were also sequenced and examined for chromosome hybridization signals. The λT240 cDNA insert mapped to the *var-1–var-2–var-3* cluster on Dd2 chromosome 7, and its sequence matched that of *var-3*. The λT244, λT284, λT287, λT288, λT295, and λT296 inserts all showed overlapping sequences and yielded the same hybridization patterns. Chromosome sites recognized by these inserts included regions within two SmaI fragments from Dd2 chromosome 7 and another from chromosome 9. We note that loss of a cytoadherence phenotype has been correlated with a chromosome 9 deletion in certain P. falciparum lines (Day et al., 1993); the possibility that *var* genes were involved in this deletion remains to be investigated.

RNA Transcripts of 1.8–2.4 kb Related to *var* Exon II

In addition to the 7.5 kb *var-7* band, a broad 1.8–2.4 kb band was detected on RNA blots after hybridization with a probe that recognizes *var* exon II (Figure 5b). Sequences of eight group II cDNA inserts homologous to exon II were therefore determined and aligned against the *var* genes (Figure 6). Comparative analysis of the insert sequences showed that all differed from one another in regions of overlap, indicating that transcription of the corresponding RNAs was from different loci. Three of the cDNA sequences (λT140, λT141, and λT148) aligned downstream of the intron–exon II splice junction. However, five other cDNA inserts (λT142, λT145, λT147, λT150, and λT152) had sequences that aligned upstream of the *var* intron–exon II splice site and included regions homologous to *var* intron sequences. In the vicinity of the splice junction, consensus splice sites occurred in three of the cDNA se-

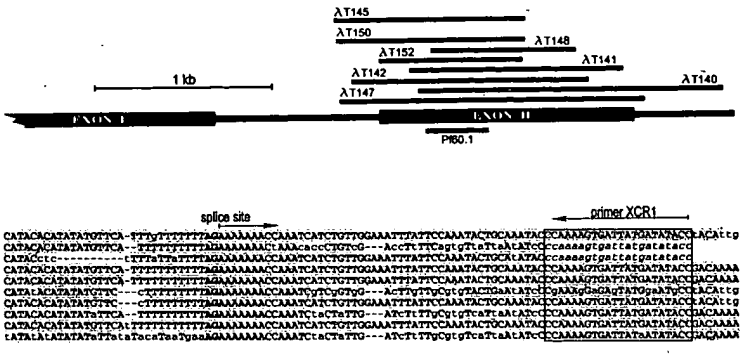


Figure 6. Alignments of Cloned cDNAs Relative to *var* Exon II

The schematic diagram shows the locations of cDNA clones λT140–λT142, λT145, λT147, λT148, λT150, and λT152 relative to the *var* gDNA structure. Sequence differences among the cDNA clones indicate that the corresponding RNAs are transcribed from different loci (data not shown). The cDNA clones have 81%–92% sequence identity to *var-1* exon II in the regions of overlap, except λT150, for which the identity is 100%. Clones λT142, λT145, λT147, λT150, and λT152 extend upstream of the intron–exon II splice site and include parts of the intron sequence. The region of homology to the Pf60.1 sequence (GenBank accession number Z28372) is indicated below exon II. Sequences in the vicinity of the splice junction are aligned at the bottom of the figure: a, *var-7* gDNA; b, *A4var* gDNA; c, *MCvar-1* gDNA; d, *var-1* gDNA; e, *var-2* gDNA; f, *var-3* gDNA; g, λT142 cDNA; h, λT147 cDNA; i, λT150 cDNA; j, λT145 cDNA. Consensus AG dinucleotides corresponding to 3′ intron splice sites are highlighted in bold. Gaps in the sequences are indicated by dashes; lowercase italics indicate the sequence of the PCR primer in the *A4var* and *MCvar-1* sequences. The region marked by the box was used to design reverse PCR primer (XCR1) for extension of the *A4var* and *MCvar-1* sequences.

quences (λT142, λT147, and λT150), while a fourth sequence (λT145) showed the required AG dinucleotide but not the expected pyrimidine tract of the splice consensus (Figure 6). The part of the fifth sequence (λT152) that aligned with the *var* intron extended upstream only to the TAG of the splice sequence. All five sequences lacked a consensus start codon preceded by AT-rich noncoding DNA that is typical of P. falciparum translation start sites.

GenBank and EMBL searches identified homology of the group II cDNA clones and *var* exon II sequences to the Pf60.1 family of transcripts reported by Carcy et al. (1994). On the basis of detection by antisera against a 316 bp sequence, the Pf60.1 family was proposed to encode a family of 60 kDa molecules in P. falciparum. The Pf60.1 probe detected transcripts having a relative size of 3 kb and hybridized to approximately 140 copies in the P. falciparum genome. While the size of 3 kb is somewhat larger than the 1.8–2.4 kb RNA band evident in Figure 5b, 140 copies is consistent with our own estimates of 50–150 *var* copies in the genome (on the basis of hybridizations with exon I and exon II probes; data not shown). No group II cDNA sequences analyzed in this work, however, were found to encode a 60 kDa protein. The intron–exon junctions and lack of consensus translation start sites instead suggest that many of these transcripts do not by themselves encode protein products.

Isolate-Specific *var* Sequences and Evidence for DNA Recombination in Cultivated Parasite Clones

The diversity of *var* forms expressed by P. falciparum parasites reflects a tremendous repertoire in the *var* gene family. This repertoire is evident in the patterns of restriction polymorphism detected by *var* probes (see Figure 2) as well as in the detection of *var*-specific sequences that hybridize to some parasite DNAs but not to others. The *var-7* gene expressed by Dd2, for example, is not present in the HB3, 3D7, or A4 genomes, whereas the *A4var* gene (Smith et al., 1995) is not found in those of Dd2, HB3, or 3D7 (data not shown). Such *var* diversity suggests that frequent DNA rearrangements underlie the production of antigenically variant types in different parasite strains.

To test for DNA rearrangements in parasites cultivated in vitro, we used *var* sequences to probe restricted DNAs from Dd2 lines adapted to neuraminidase-treated erythrocytes (Dolan et al., 1990). Figure 7 shows evidence for two different rearrangements in these lines. In one rearrangement, a novel 35 kb BglI fragment is seen in NM1 DNA probed with the λT142 (group II) insert. In another rearrangement, a deletion of a 20 kb PstI band is evident in NM8 DNA probed with a *var-7* sequence. Deletion of this 20 kb band was also detected in the Dd2/R8 subclone obtained before neuraminidase selection (Dolan et al., 1990), indicating that the DNA rearrangement was not produced by selection in neuraminidase-treated erythrocytes (data not shown).

Comparative Analysis of *var* Genes Implicated in the Antigenic Variation and Cytoadherence of P. falciparum-Infected Erythrocytes

The accompanying papers by Smith et al. (1995) and Baruch et al. (1995) provide direct evidence for the involvement of DBL domains in the antigenic variation and cytoadherence of P. falciparum–infected erythrocytes. Smith et al. (1995) show that switches in transcription of these genes correlate with changes in the antigenic determinants and ICAM-1-binding phenotypes of cloned parasite lines. Four different DBL sequences have been associated with unique antigenic and cytoadherence phenotypes, of which three (encoded by *C18var*, *R29var*, and *C24var*) have the signature of DBL domain 1, and one (encoded by *A4var*) has the signature of DBL domain 3. In another strategy, Baruch et al. (1995) have obtained a 3.5 kb cDNA sequence from a P. falciparum MC[K+] transcript (*MCvar-1*, clones A1–D2). Using recombinant proteins corresponding to regions of this cDNA sequence, these authors generated antisera that immunoprecipitate a protein with the characteristics of MC[K+] PfEMP1, react in a strain-specific manner over knob protrusions on MC[K+]-parasitized erythrocytes, and block the adherence of MC[K+]-parasitized

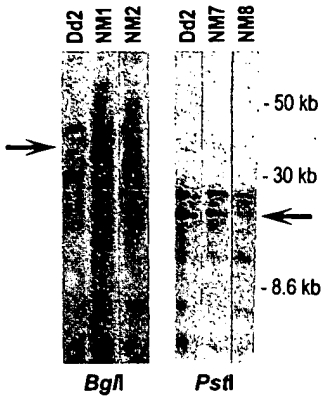


Figure 7. Evidence for *var* Rearrangements in Parasite Lines Derived from the Dd2 P. falciparum Clone

Lines NM1, NM2, NM7, and NM8 were obtained from Dd2 parasites propagated in neuraminidase-treated red cells (Dolan et al., 1990). Left, a new 35 kb fragment (arrow) in NM1 parasites. DNA was digested with BglI and probed with the λT142 cDNA insert. Right, deletion of a 20 kb band (arrow) from NM8 parasites. DNA was digested with PstI and probed with the c242-3'P4 insert containing nt 4681–6504 of the *var-7* cDNA sequence. Deletion of the 20 kb band was also detected in the Dd2/R8 subclone that is the NM8 precursor (data not shown).

erythrocytes to CD36. The amino acid sequence encoded by this MC[K+] cDNA sequence contains DBL domain 1, the CIDR, and a portion of DBL domain 2.

To confirm the relatedness of these A4 and MC[K+] cDNA sequences to the *var* family, we designed specific oligonucleotide primers from the data of Baruch et al. (5'-TCCCCATCTCCTCCTCGTGTC-3') and Smith et al. (5'-AGTGAAAAAGCAACAGAAACTGAATTG-3'), paired these with reverse primer XCR1 (5'-GGTATATCATAA[A/T]CACTTTTGG-3') from a conserved region of exon II (boxed region, Figure 6), and amplified the genomic sequences from A4 and MC[K+] parasite DNAs. The products from these amplifications (a 3.6 kb A4–genomic DNA [gDNA] fragment and a 5 kb F-gDNA fragment, respectively) were cloned, sequenced, and found to overlap perfectly at their 5' end with the A4 and MC[K+] cDNA sequences (0.5 kb and 0.12 kb overlaps, respectively; Smith et al., 1995; Baruch et al., 1995). Analysis of each composite sequence showed two ORFs interrupted by a ~1 kb intron typical of *var* genes. Figure 4 includes the amino acid alignments from these ORFs. The extended *A4var* sequence includes part of domain 3, domain 4, the putative transmembrane segment, and 15 amino acids encoded by the exon II. The *MCvar-1* composite sequence includes the start methionine, four DBL domains, a transmembrane segment, and 16 amino acids encoded by exon II. These features confirm that the A4 and MC[K+] cDNA sequences are from genes of the *var* family.

Discussi n

Evidence presented in this and accompanying reports (Smith et al., 1995; Baruch et al., 1995) shows that genes of the P. falciparum *var* family encode 200–350 kDa variant surface molecules that determine antigenic and adhesive properties of parasitized rythrocytes. The larg repertoire of *var* genes (50–150 copi s, having sufficient DNA to account for 2%–6% of the haploid genome), the dramatic sequence variation among the gene copies, their variable expression in different parasite lines, the ready detection of DNA rearrangements, and the receptor binding features of the encoded extracellular domains all implicate *var* genes as the major determinants of antigenic variation and cytoadherence in P. falciparum malaria.

The *var* genes encode multiple extracellular domains that are homologous to the cysteine-rich binding domains of certain Plasmodium molecules involved in the invasion of erythrocytes. These include the binding domains of the P. falciparum glycophorin A recognition molecule EBA-175 and the P. vivax and P. knowlesi ligands that determine invasion of Duffy blood group–positive erythrocytes (Adams et al., 1992; Chitnis and Miller, 1994; Sim et al., 1994). Such homology suggests that genes encoding these domains can be grouped into a superfamily, which we propose terming the Duffy binding–like or DBL superfamily, after the binding domain of the P. vivax ligand. In P. falciparum, the DBL superfamily would include single copy, well-conserved genes such as *eba-175* and *ebl-1* (Peterson et al., 1995) as well as the multicopy and highly polymorphic *var* genes. The occurrence of DBL domains in proteins encoded by such diverse genes suggests that the basic DBL structure has been adapted to many different adhesive and receptor recognition functions in malaria.

The organization of the DBL domains encoded by the *var* genes is reminiscent of that of other extracellular domains in such cell–cell adhesion molecules as cadherins and members of the immunoglobulin superfamily (Hynes, 1994; Overduin et al., 1995). Extracellular domains in these molecules are typically arranged as imperfect repeats, with binding specificity being generally determined by the N-terminal domain away from the cell surface. This suggests that the conserved head structure formed by DBL domain 1 and the DBL-related CIDR may contain extracellular sites that are largely responsible for adhesive specificity. The analogy with other cell–cell adhesion molecules also raises the possibility that P. falciparum variant surface molecules may not merely use host cell receptors such as CD36, ICAM-1, VCAM-1, and ELAM-1 as inert attachment sites. Binding to these cell receptors may induce complex signaling and secretion events (Luscinskas and Lawler, 1994) that could influence the course of malaria and affect severe manifestations of the disease (e.g., cerebral malaria).

Sequence diversity of the *var* genes is most evident in th regions in and around the DBL and CIDR domains. This is consistent with th ne d for the parasite to vary extracellular regions of the surfac molecul s that ar accessible to immune attack. The cons rv d amino acid residues in the DBL and CIDR domains may maintain the folding necessary for adhesion and r c ptor function, while allowing extrem variations in xposed r gions that provide immune evasion. We note that conservation of

structure in antigenically variant molecules has been demonstrated for the variant-specific surface glycoproteins (VSGs) of African trypanosomes. Crystallographic comparison of two different VSG N-terminal variable domains showed that the domains had nearly the same three-dimensional structures, despite dramatic differences in amino acid sequences (Blum et al., 1993).

The predicted amino acid sequences from the *var* genes show a putative transmembrane sequence followed by an acidic terminal segment. This segment may serve in localizing the variant surface molecule at the surface of infected erythrocytes. Immunoelectron microscopy has shown that the reactivity of variant-specific antibody is concentrated over knob protrusions that are introduced by the parasite into the surrounding erythrocyte membrane (Langreth and Reese, 1979; Baruch et al., 1995). Is there an association between the variant surface molecules and molecules in the knob structures? Subcellular accumulations of a highly charged histidine- and lysine-rich protein (KP) are present in knobs (Sharma and Kilejian, 1987; Pologe et al., 1987; Triglia et al., 1987; Ardeshir et al., 1987; Ellis et al., 1987). The KP histidine and lysine residues may form salt bridges with the acidic terminal segments and thereby anchor the variant surface molecules at the knobs.

An unusual family of 1.8–2.4 kb RNA transcripts cross-hybridizes with the conserved 3′ sequences of the *var* genes. Sequences of several cDNA clones corresponding to these transcripts were found to align not only with *var* exon II, but with the *var* intron as well. The presence of *var* intron sequences and the lack of consistent initiation methionines suggests that many of these 1.8–2.4 kb RNAs do not encode protein products on their own. The relative abundance of these transcripts also suggests that they do not merely derive from the breakdown of full *var* transcripts. We speculate that the transcripts may participate in expression or rearrangements of *var* genes. The pool of small transcripts could, for example, be involved in *trans*-splicing events that have regulatory importance, or there may be initiation from internal promoters that could affect transcription from *var* upstream regions. Another hypothesis is that transcription of the 1.8–2.4 kb RNAs may correlate with accessibility of *var* genes to DNA rearrangements. Transcription activity stimulates recombination in certain yeast and mammalian genes (Thomas and Rothstein, 1989; Nickoloff and Reynolds, 1990), and transcription is thought to be a correlate of rearrangement activity in the immunoglobulin and T cell receptor loci of lymphocytes (Alt et al., 1987; Okada and Alt, 1994).

Frequent antigenic variation in Plasmodium has been noted to resemble the switches in VSG expression that occur in African trypanosomes (Brown and Brown, 1965). Indeed, in their sequence diversity and variable expression, the *var* genes are analogous to the trypanosome *vsg* genes, but already differences are evident in the way *var* and *vsg* genes are organized and expressed. Expression of trypanosome *vsg* genes is from telomeric sites, and a major route of antigenic variation is thought to involve DNA rearrangements that duplicate sequences from a chromosome-internal repertoire of genes into these sites. By contrast, P. falciparum *var* genes do not appear to require duplicative transpositions to telomeric sites for expression. The exact nature and location of *var* expression sites in P. falciparum remain to be determined.

As evidence builds for the role of *var* genes in the antigenic variation and cytoadherence of P. falciparum–infected erythrocytes, significant questions include the pathways by which the variant surface proteins are targeted to the erythrocyte surface, the sites that determine adhesive specificity, and the mechanisms responsible for sequence diversity and switches in expression of *var* genes. The *var* genes are responsible for large numbers of serotypically different forms, a diversity that is probably enhanced greatly by the frequent genetic recombination that occurs among parasites in regions of high malaria transmission (Babiker et al., 1994). Investigations of these questions will advance our understanding of malaria and perhaps foster innovative approaches to vaccine development or therapeutics.

Experimental Procedures

Parasite Clones, DNA Analysis, and Chromosome Mapping

Parasite clones were cultivated by the methods of Trager and Jensen (1976). DNA was extracted from parasite cultures as described (Peterson et al., 1988), except that the DNA was recovered by ethanol precipitation rather than spooling. Fingerprint analysis with the pC4.H32 probe was used to confirm DNA preparations (Dolan et al., 1993). Southern blotting to Nytran membranes was as recommended by the manufacturer (Schleicher and Schuell, Keene, NH). PFG separation of the 14 P. falciparum chromosomes and chromosome mapping were performed as described (Wellems et al., 1987; Sinnis and Wellems, 1988).

RNA Isolation

Parasites from 200 ml mixed stage cultures (5%–10% parasitemia) were released by saponin lysis as for DNA preparations, except that the procedures were performed with ice-cold solutions. RNA was immediately isolated from the parasite pellet by guanidine thiocyanate/phenol–chloroform methods, recovered, and treated with RNase-free DNase (Creedon et al., 1994). RNA in H_2O was combined with 2 vol of 100% EtOH, distributed into 2 ml vials, and frozen as stock at −70°C. RNA was recovered by precipitation with 0.1 vol of 3 M NaOAc. RNA blots were generated and probed as described (Creedon et al., 1994).

YAC Isolation, Chromosome Segment Libraries, and cDNA Libraries

Overlapping YACs spanning the 300 kb segment of chromosome 7 that contains the CQR locus were obtained from a YAC library of a CQR FCR3 parasite line (de Bruin et al., 1992) by the procedures of Lanzer et al. (1993). Orientation of the YACs and their overlaps were identified with probes obtained from the YAC ends by inverted PCR.

Attempts to construct cosmid libraries and large insert (~10 kb) λ libraries from high molecular weight P. falciparum genomic DNA yielded only rearranged clones. An alternative approach was therefore taken in which chromosome-segment libraries were constructed that contained small (0.5–5 kb) inserts in plasmid vectors. Plasmid libraries containing AluI, HinfI, RsaI, and SspI inserts in pCDNAII were produced from Dd2 chromosome 7 restriction fragments purified by PFG electrophoresis (Wellems et al., 1991). A plasmid library from a 34 kb ApaI–SmaI restriction fragment of YAC PfYED9 was constructed by the same methods.

The λgt10 Dd2 cDNA library was prepared under contract by CloneTech Laboratories, Incorporated (Palo Alto, CA) from the DNase-treated, poly(A)$^+$ fraction of Dd2 RNA. The cDNA was generated in two separate reactions using oligo(dT) primers or random primers.

Products of these reactions were combined, processed, and cloned into the EcoRI site of λgt10. Independent recombinants (1.6×10^6) were obtained and amplified.

Isolation of Overlapping Clones and DNA Sequencing

Plasmid clones from the chromosome-segment and YAC-segment libraries were picked at random, and their locations were established by restriction mapping. After sequence data from these clones were generated, overlapping clones were isolated in a process of chromosome walking by rescreening the libraries with oligonucleotide probes near the ends of sequenced inserts. Sufficient divergence was present among repetitive elements in the sequences to allow distinction of clones and unambiguous assignment of overlaps (generally 50–200 bp).

Sequencing reactions with single-strand M13 DNA (1 μg) or double-strand plasmid DNA (2–5 μg) were performed in 96-well polyvinyl chloride U-bottom microassay plates according to a Sequenase protocol recommended by United States Biochemical Corporation (Cleveland, OH). Reactions were separated by 8 M urea–6% polyacrylamide sequencing gels and exposed to Kodak BioMax MR film. Sequence data from some clones were also obtained by use of an ABI 373A automated DNA sequencer (Applied Biosystems, Incorporated, Foster City, CA). Cycle sequencing reactions were performed with the Applied Biosystems PRISM DyeDeoxy system.

DNA sequence editing, analyses, and display were performed with MacVector software (International Biotechnologies Incorporated, New Haven, CT), BLAST (Altschul et al., 1990), Genetics Computer Group programs (Devereux et al., 1984), and the DNADRAW package (Shapiro and Senapathy, 1986) maintained at the National Institutes of Health.

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GenBank Accession Numbers

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